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WO 96/16162 A1 WO 96/04368 A1 WO 94/09110 A1
WO 94/04658 A1 WO 93/17696 A1 WO 93/17045 A1
WO 93/05807 A1 WO 92/01039 A1 WO 91/09936 A1
WO 91/07992 A1
MOLECULAR ENDOCRINOLOGY, Vol. 7, No. 2, 1993, pages 189-198 PROC.NATL.ACAD.SCI., Vol. 74, No. 3, 1977, pages 1200-1203

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UK CL (Edition O) C6F FHC1 FX
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ONLINE: WPI; BIOTECH/DIALOG

(54) Method for controlling differentiation of precursor cells

(57) There is detailed a method for regulating or controlling the differentiation of precursor cells to at least one specific phenotype comprising exposing the cells to blood or neural cell milieu or an extract thereof so as to selectively drive differentiation to a predetermined mature phenotype. Examples of the extracts include prostaglandins, retinoic acid, neurotrophic factors (e.g. CNTF, FGF, BDNF), fibroblast growth factor, hydrocortisone, dexamethasone. Also claimed are agents which inhibit this differentiation.

GB 2 301 114 A

Figure 1.

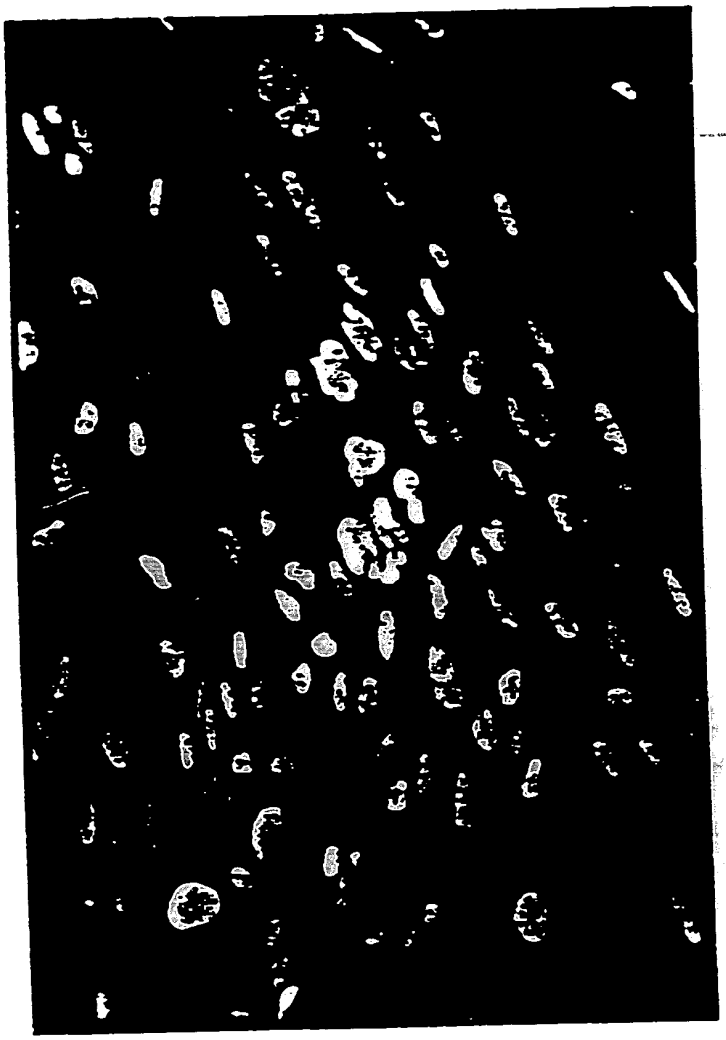
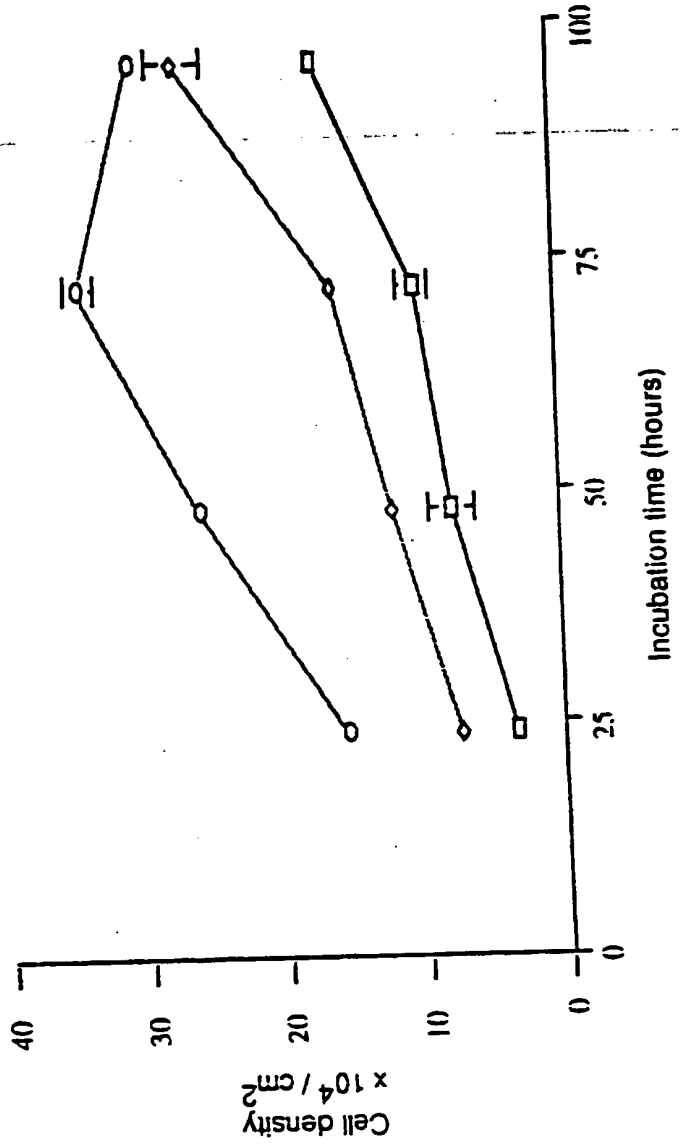
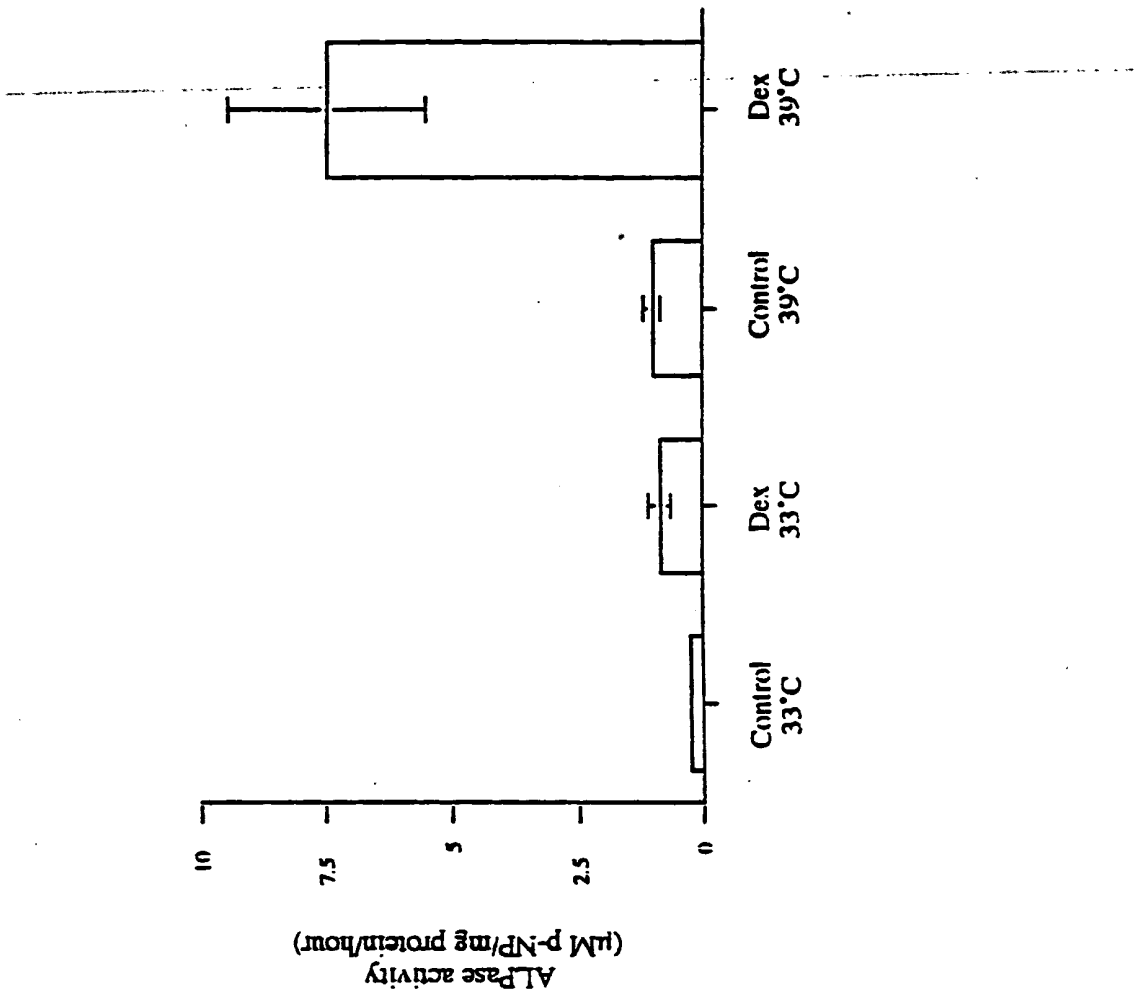


Figure 2.


Cell density
x 10⁴ / cm²
40
30
20
10
0
0
25
50
75
100
Incubation time (hours)

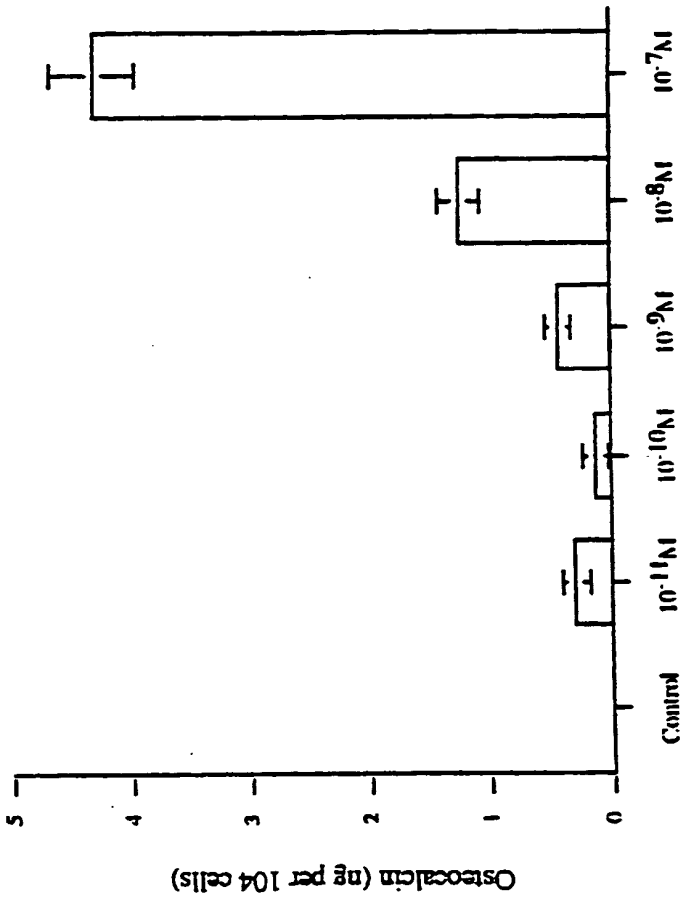
3/12

Figure 3a.



4112

Figure 3b.



5/12

Figure 4.

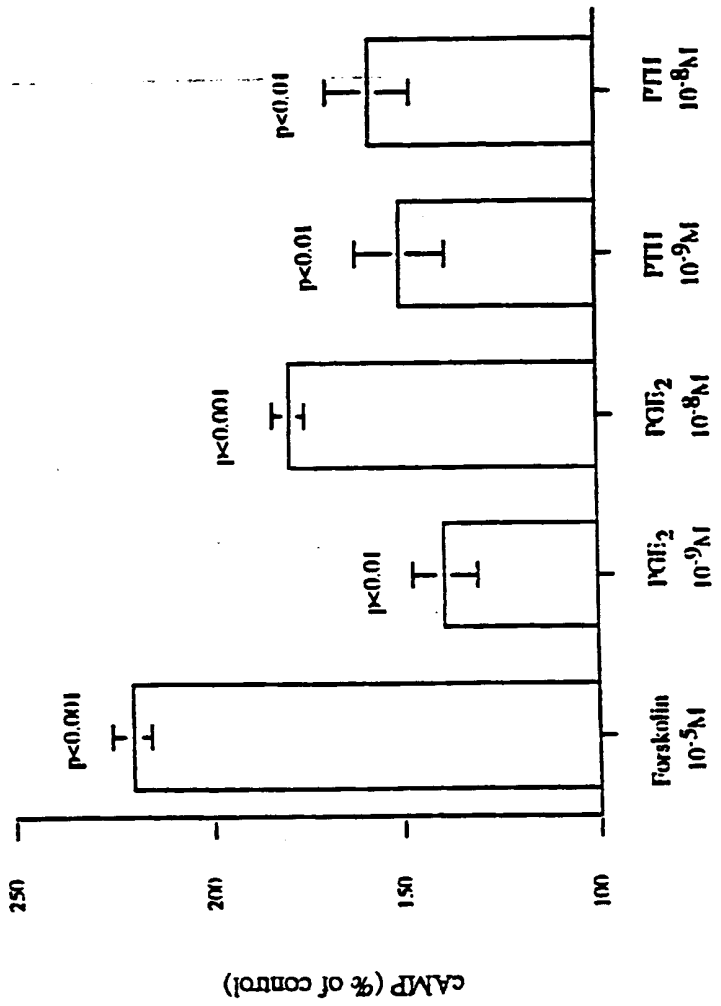

cAMP (% of control)
250
200
150
100
Forskolin 10^{-5}M
p<0.001
PGI$_2$ 10^{-9}M
p<0.01
PGI$_2$ 10^{-8}M
p<0.001
PTII 10^{-9}M
p<0.01
PTII 10^{-8}M
p<0.01

Figure 5.

Figure 6.

8/12

Figure 7a.

Figure 7b.

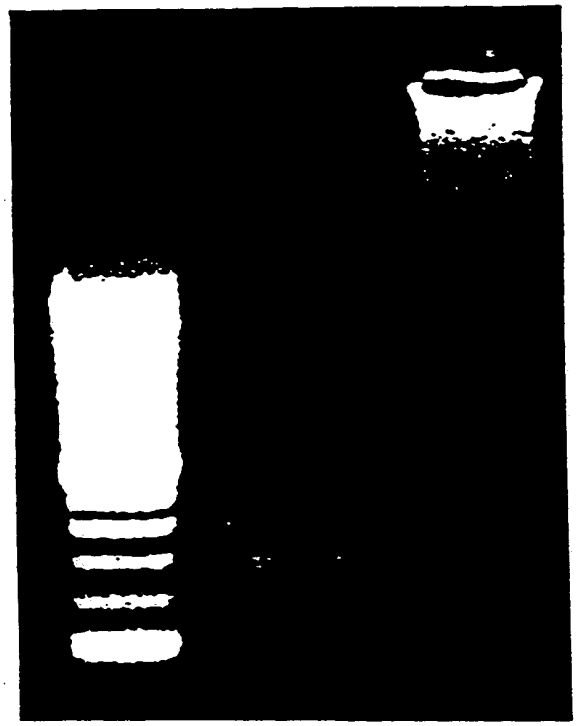


Figure 8.

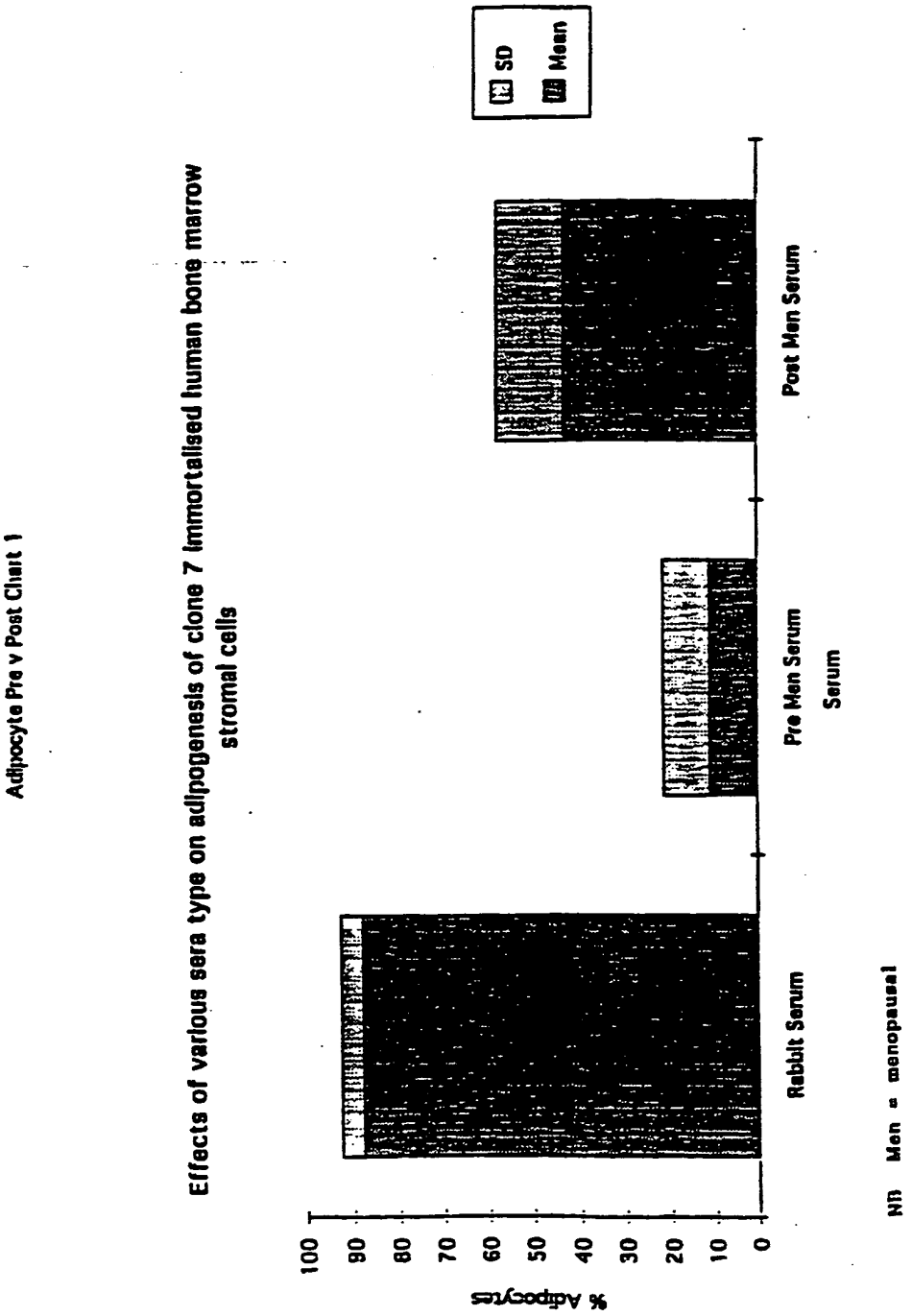

Adipocyte Pre v Post Chart 1
Effects of various sera type on adipogenesis of clone 7 immortalised human bone marrow stromal cells
SD
Mean
% Adipocytes
100
90
80
70
60
50
40
30
20
10
0
Rabbit Serum
Pre Men Serum
Post Men Serum
Serum
NB Men = menopausal

Figure 9.

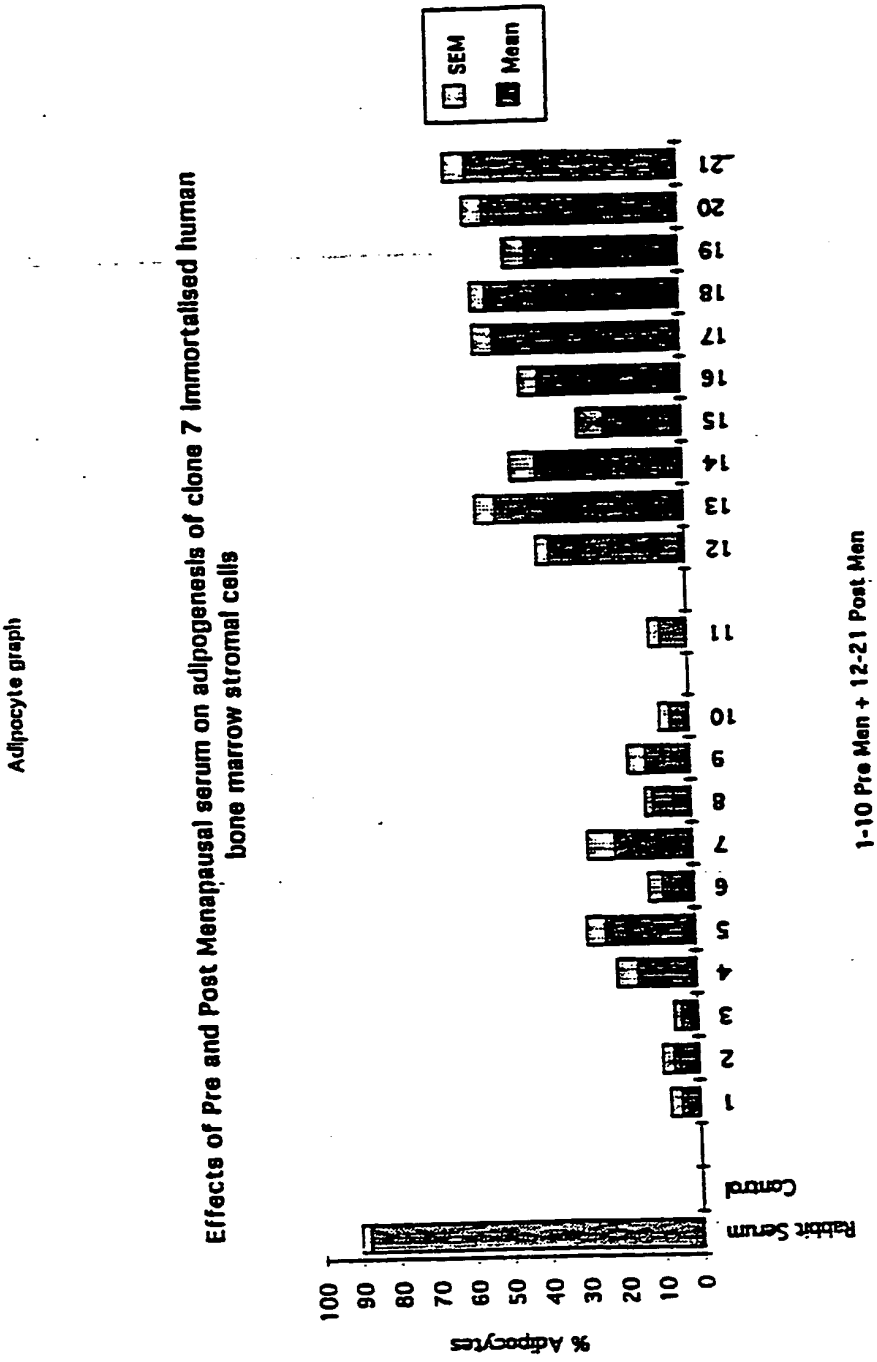
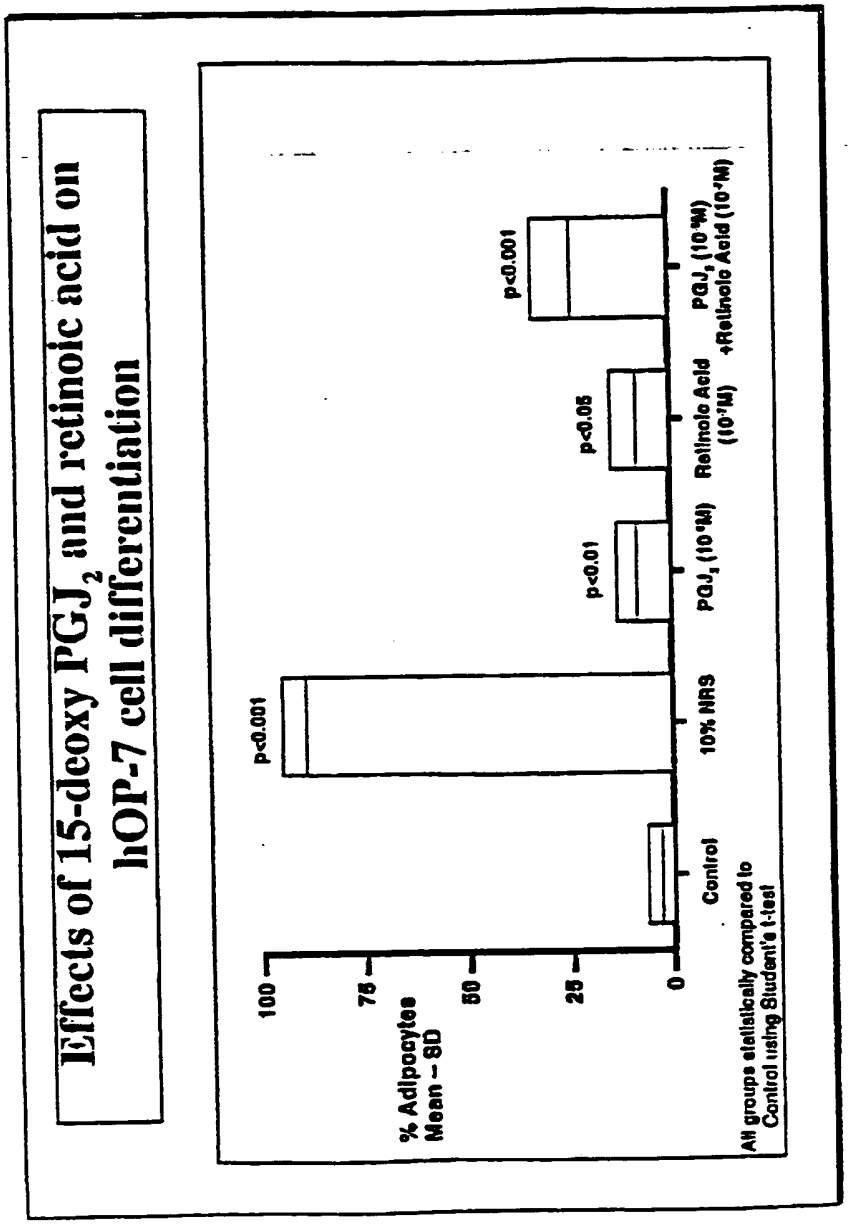

Effects of Pre and Post Menapausal serum on adipogenesis of clone 7 immortalised human bone marrow stromal cells
Adipocyte graph
SEM
Mean
% Adipocytes
100
90
80
70
60
50
40
30
20
10
0
Rabbit Serum
Control
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
1-10 Pre Men + 12-21 Post Men

Figure 10


Effects of 15-deoxy PGJ_2 and retinoic acid on hOP-7 cell differentiation
% Adipocytes Mean – SD
100
75
50
25
0
p<0.001
p<0.01
p<0.05
p<0.001
Control
10% NRS
PGJ_2 (10^{-6}M)
Retinoic Acid (10^{-7}M)
PGJ_2 (10^{-6}M) +Retinoic Acid (10^{-7}M)
All groups statistically compared to Control using Student's t-test

DIFFERENTIATION AGENTS

The invention relates to agents that affect the differentiation of cells; methods including such agents and products including such agents.

Cellular differentiation is a process whereby cells become different from one another, they specialise so as to perform particular functions. Thus, for example, cells of the retina become specialised so that they can respond to light and convey this response to the brain, alternatively, cells of the skeleton become specialised so that they can either manufacture bone (osteoblasts) or cartilage (chondrocytes), or alternatively resorb bone (osteoclasts).

The nature of events that trigger the differentiation pathway and the nature of the markers that define each differentiation pathway are currently under investigation. Perturbations in the differentiation process clearly will have significant consequences for a developing embryo and a young human or animal, or indeed any developing organism.

For tissue that is actively being turned over throughout life, perturbations in the differentiation process can lead to disease. For example, it is known that bone is actively turned over throughout life and defects in this turnover can lead to skeletal disorders such as osteoporosis. Since bone is produced by osteoblasts and resorbed by osteoclasts attention has been focused on the differentiation of these two cell types because clearly a preponderance towards one or other cell type would affect the balance in bone turnover. However, there are very few markers of the cell differentiation process and thus current knowledge is scant. Despite this fact, it has been proposed that osteoblast differentiation from a mesenchymal stem cell occurs either as a

restriction in the multipotentiality of early precursor cells during maturation, or due to commitment to a differentiation pathway, or cell lineage, early in the mesenchymal stem cell development, but knowledge of this process is scant.

Indeed, it is thought, that differentiated cells are preceded by precursor cells which at some stage become committed to a particular differentiation pathway so as to provide a fully differentiated phenotype. However, the teaching encompasses the idea of multipotentiality and thus it is thought that, depending upon the circumstances, a precursor cell can produce a number of different phenotypes. Unfortunately, the nature of the circumstances remain largely obscure.

It can be seen from the above that a greater understanding of the differentiation process would be advantageous and would help to safeguard against or mitigate deleterious consequences associated with perturbations in differentiation, typically by way of ensuring a particular differentiation pathway is undertaken and so safeguarding against disease conditions associated with a lack of products resulting from said pathway.

In order to investigate cellular differentiation we have used immortalised cell lines and in particular immortalised human cell lines as described in our co-pending UK patent application 9522562.9. These cell lines have been provided by immortalising human precursor cells, such as human bone marrow stromal cells, using retroviral transduction with a modulatable oncogene. This is advantageous because immortalisation is induced by a single genetic event, hence the genotype, and, consequently, the phenotype of such cell lines are clearly defined. Moreover, expression of the active

form of the immortalising oncogene product is controllable. Thus, once a large, homogeneous population of precursor cells of interest has been produced, the oncogene protein can be inactivated so allowing the cells to return to their original state of differentiation. Using these cells we have shown that cellular differentiation does involve plasticity in that precursor cells have multipotentiality and thus can differentiate down a number of different differentiation pathways so as to provide for different cell phenotypes having regard to the nature of the pathway undertaken. This multipotentiality is controlled by an external agent acting upon the precursor cells and more specifically we have found it is controlled by blood, or an extract thereof and/or neural cell extracellular milieu, or an extract thereof. This observation has significant ramifications because it clearly suggests that by controlling the environment to which a precursor cell is exposed one can control the nature of the mature phenotype.

Thus, we suggest for the first time that the multipotentiality of precursor cells can be exploited to advantage and particularly, but not exclusively, in the treatment of clinical conditions.

In just one example, we have observed that human bone precursor cell lines will differentiate to the osteoblast phenotype under normal culture conditions, these conditions include exposure to dexamethasone/$(OH)_2$Vitamin D_3 administration, but when exposed to blood and particularly serum, or an extract thereof, these same human bone precursor cell lines differentiate to an adipocyte phenotype. This observation accords well with the fact that the volume of marrow adipose tissue is significantly increased and bone volume reduced in primary osteoporotic and osteopenic patients of all ages. In addition, it is also known from animal studies that an increase in the volume

of marrow fat occurs after the reduction of bone loss by: immobilisation, exposure to zero gravity, or ovariectomy. To date, no cell related mechanism has been identified which may account for the inverse relationship between the volume of marrow adipose tissue and bone.

Thus we have realised that osteoporosis or the increase in volume of marrow adipose tissue, can be treated by using our human cell lines to identify agents that block the activity of blood and particularly serum, or an extract thereof, and then using such agents *in vivo*.

This is the first time that anyone has thought to exploit the multipotentiality of precursor cells to provide treatments of clinical significance. Moreover, it is also the first time that anybody has manufactured human cell lines with a view to using them to identify agents that exploit the multipotentiality of precursor cells by driving these precursor cells along selected differentiation pathways.

In an alternative example, we have observed that human neural cell precursor cell lines will differentiate to either the nerve cell phenotype under normal culture conditions, these conditions include exposure to glial cell line-derived neurotrophic factor (GDNF), but when exposed to serum, or an extract thereof, the same human neural cell precursor cell lines differentiate to a glial such as an astrocyte phenotype.

These findings suggest that diseases characterised by a lack of neural cell tissue can be treated by using our human cell lines to identify agents that block the activity of serum, or an extract thereof, and then using such agents *in-vivo*.

It is therefore an object of the invention to provide methods and means for regulating or controlling, or predicting, the differentiation of precursor cells.

In its broadest aspect the invention concerns the exploitation of the multipotentiality of precursor cells in order to selectively drive differentiation along a pre-determined pathway with a view to selecting the nature of the mature phenotype.

According to a first aspect of the invention there is therefore provided a method for regulating or controlling the differentiation of precursor cells to at least one specific phenotype comprising exposing said cells to blood and particularly serum, or an extract thereof, and/or neural cell extracellular milieu, or an extract thereof, under conditions that support differentiation so as to selectively drive differentiation with a view to providing a preselected mature phenotype.

According to a second aspect of the invention there is provided a method for regulating or controlling the differentiation of precursor cells to at least one specific phenotype comprising exposing said cells to an agent that blocks the activity of blood and particularly serum, or an extract thereof, and/or neural cell extracellular milieu, or an extract thereof, under conditions that support differentiation.

In the first aspect of the invention said blood and particularly serum, or an extract thereof, and/or said milieu, or an extract thereof, is used to induce, what may be termed, serum directed or neural cell extracellular milieu directed differentiation, respectively, so as to provide a serum or neural cell extracellular milieu directed phenotype.

In the second aspect of the invention said agent is used to block what we have termed serum and/or neural cell extracellular milieu directed differentiation so as to provide for an alternative phenotype which may be termed a serum or nerve cell extracellular milieu independent phenotype.

According to a third aspect of the invention there is provided a pharmaceutical composition comprising an effective amount of: blood and particularly serum, or an extract thereof, and/or neural cell extracellular milieu, or an extract thereof, for directing differentiation of selected precursor cells to at least one specific phenotype.

According to a fourth aspect of the invention there is provided a pharmaceutical composition comprising an effective amount of agent that blocks the activity of blood and particularly serum, or an extract thereof, and/or neural cell extracellular milieu, or an extract thereof, so as to provide for serum or neural cell extracellular milieu independent differentiation of selected precursor cells to at least one serum or neural cell extracellular milieu independent phenotype.

In the third aspect of the invention said composition may be used in relation to bone precursor cells so as to provide for a an adipocyte phenotype; or in relation to neural precursor cells so as to provide for a glial phenotype, so safeguarding against diseases such as multiple sclerosis which is characterised by a reduction in oligodendrocytic glial cells.

In the fourth aspect of the invention said pharmaceutical composition may be used in relation to bone precursor cells so as to provide for an osteoblast phenotype so safeguarding against osteoporosis, osteopaenia, osteoarthritis,

bone fractures, rickets and other skeletal conditions associated with a reduction in bone formation, and furthermore obesity and other conditions associated with an increase in adipose tissue and for (cardio) vascular disease whereby the build up of fat (cells) in the vascular system may lead to changes in blood flow. Moreover, a modification of differentiation is relevant to muscle wastage in aged individuals. In the instance where the fourth aspect of the invention is used in relation to neural precursor cells it provides for an increase in production of neuronal cells and thus may be used to safeguard against diseases associated with a reduction in neuronal cells such as stroke injury, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis or Motor Neurone Disease or Huntingtons chorea.

According to a fifth aspect of the invention there is provided a method of preventing, mitigating or alleviating osteoporosis or obesity comprising administering to an individual to be treated a pharmaceutical composition comprising an effective amount of a blood and, particularly a serum, and/or neural cell extracellular milieu blocking agent that blocks serum and/or neural cell extracellular milieu directed differentiation so as to provide for a bone related phenotype.

Ideally said phenotype is an osteoblast phenotype.

In a preferred embodiment of the invention said pharmaceutical composition is administered to a site to be treated.

According to a sixth aspect of the invention there is provided a method for promoting or enhancing the amount of adipose tissue in an individual to be treated comprising administering to said individual a pharmaceutical

composition comprising an effective amount of serum, or an extract thereof and/or neural cell extracellular milieu or an extract thereof, to induce serum and/or neural cell extracellular milieu directed differentiation of an adipose cell precursor cell so as to provide for a serum directed adipose phenotype.

Where the invention is to be used in respect of neural cell precursor cells we have found that nerve cell extracellular milieu can selectively drive differentiation so as to produce what we have termed neural cell extracellular milieu directed differentiation and a nerve cell extracellular milieu phenotype.

According to a seventh aspect of the invention there is provided a method for preventing, mitigating or alleviating diseases associated with a reduction in neuronal cells comprising administering to an individual to be treated a pharmaceutical composition comprising an effective amount of agent that blocks serum directed differentiation and/or neural cell extracellular milieu directed differentiation and so ensures that neural cell precursor cells differentiate in a serum and/or neural cell extracellular milieu independent manner so as to provide for a neuronal cell phenotype.

In a preferred embodiment of the invention the pharmaceutical composition is directed towards the tissue of the central nervous system.

In this aspect of the invention the pharmaceutical composition provides for an increase in the production of neuronal cells and thus may be used to safeguard against diseases associated with a reduction in neuronal cells such as stroke injury, Alzheimer's disease, Parkinson's disease, amytrophic lateral sclerosis or Motor Neurone Disease or Huntingtons chorea.

According to an eighth aspect of the invention there is provided a method of identifying agents effective at preventing or interfering with serum directed differentiation and/or neural cell extracellular milieu directed differentiation comprising: exposing a precursor cell population to an effective amount of

a) blood and particularly serum, or an extract thereof, and/or an effective amount of neural cell extracellular milieu, or an extract thereof; and/or

b) a test agent

for an effective amount of time under conditions that support differentiation and then examining said population to determine whether and/or to what degree serum or neural cell extracellular milieu directed differentiation has taken place.

This method of the invention may be performed *in vitro* or *in vivo*.

In a preferred embodiment of the invention said cell population is a cell line and ideally of human origin such as a human bone marrow stromal cell line or a human neural cell line.

Clearly agents identified using this method of the invention would be effective at blocking serum and/or neural cell extracellular milieu directed differentiation and so when used in relation to bone precursor cells would provide for the production of a bone related phenotype such as an osteoblast phenotype, and when used in relation to neural cell precursor cells would provide for the production of neuronal cells.

According to a yet further aspect of the invention there is provided an agent identified by the afore method of identification.

According to a yet further aspect of the invention there is provided a method of identifying agents that are effective at enhancing serum and/or neural cell extracellular milieu directed differentiation comprising: exposing a precursor cell population to an effective amount of

a) blood and particularly serum, or an extract thereof, and/or an effective amount of neural cell extracellular milieu or an extract thereof; and/or

b) a test agent

for an effective amount of time under conditions that support differentiation and then examining said population to determine whether and/or to what degree serum or neural cell extracellular milieu directed differentiation has taken place.

This method of the invention may be performed *in vitro* or *in vivo*.

In this preferred method of the invention said cell population is a cell line ideally of human origin such as a human bone marrow stromal cell line or a human neural cell line.

According to a yet further aspect of the invention there is provided an agent identified by the afore method of identification.

Agents identified using this method of the invention when used in relation to

bone precursor cells provide for enhanced differentiation to an adipose phenotype, and when used in relation to neural precursor cells provide for enhanced differentiation to a glial, including an astrocyte phenotype.

According to yet a further aspect of the invention there is provided a method for enhancing bone formation and, at least relatively, reducing adipose formation comprising exposing a bone precursor cell population to an agent effective at blocking serum and/or neural cell extracellular milieu directed differentiation of said precursor cells to an adipose phenotype.

In a preferred embodiment of this aspect of the invention the relative balance between bone tissue and adipose tissue may be reversed by removing said agent and/or exposure to serum, or an extract thereof, and/or neural cell extracellular milieum, or an extract thereof, which promotes directed differentiation to adipose tissue.

This method may be performed either *in-vivo* or *in-vitro*.

In yet a further aspect of the invention there is provided a method for enhancing the formation of neuronal cell tissue and, at least relatively, reducing the amount of glial or more specifically astrocyte tissue comprising exposing a neural precursor cell population to an agent effective at blocking serum and/or neural cell extracellular milieu directed differentiation so that said population differentiates to a neuronal cell phenotype.

In a preferred embodiment of this aspect of the invention the balance between neuronal tissue and astrocyte or oligodendrocyte tissue may be reversed by removing said blocking agent and/or exposing said population to serum, or

an extract thereof, and/or neural cell extracellular milieu, or an extract thereof; which provides for directed differentiation so as to produce a glial or more specifically an astrocyte phenotype.

This method may be performed either *in vivo* or *in vitro*.

Reference herein to blood and particularly serum, and an extract thereof, includes reference to a diverse range of serum, such as, but not limited to human, rabbit, foetal calf, new born calf, foetal horse, adult horse, goat and donkey.

Further, reference herein to blood and particularly serum, and an extract thereof, includes reference to prostaglandins and in particular prostaglandin metabolites such as prostaglandin J2 and its metabolites, and/or retinoic acid and/or to various trophic factors such as, but not limited to, ciliary neurotrophic factor CNTF, glial cell-line derived neurotrophic factor GDNF, fibroblast growth factor FGF and other molecules such as hydrocortisone, and indeed analogues and/or homologues of the aforementioned agents.

Reference herein to neural cell extracellular milieu, or an extract thereof includes reference to a diverse range of such milieu, such as, but not limited to, human, rabbit, foetal calf, new born calf, foetal horse, adult horse, goat and donkey.

Further, reference herein to neural cell extracellular milieu, and an extract thereof, includes reference to various factors found in said milieu such as, but not limited to, ciliary neurotrophic factor CNTF, and/or glial cell-line derived neurotrophic factor GDNF, and/or brain derived neurotrophic factor BDNF,

and/or retinoic acid and/or steroid hormones such as hydrocortisone or dexamethasone, and indeed analogues and/or homologues of the aforementioned agents.

Reference herein to agents effective at blocking serum and/or neural cell extracellular milieu includes reference to agents specifically blocking the activity of CNTF, retinoic acid, FGF and PG_{J2s}.

Indeed, in further experiments we have shown that for osteoblast/adipocyte precursor cells the serum directed effect can be removed by charcoal stripping suggesting that a low molecular weight component is responsible for the directed differentiation. In addition, we have also shown that this directed differentiation can be blocked by removing molecules greater than 30Kd and more specifically 100Kd. This tends to suggest that there exists a small molecular weight differentiation agent which is bound to a large carrier protein.

According to a further aspect of the invention there is therefore provided a method of identifying an extract that is effective at providing serum directed differentiation comprising fractionating blood and particularly serum and then exposing a precursor cell population to an effective amount of at least one fraction of said serum for an effective amount of time under conditions that support differentiation and then examining said population to determine whether and/ or to what degree serum directed differentiation has taken place.

This method may be performed *in vitro* or *in vivo*.

A further aspect of the invention comprises an extract identified by the afore

method.

According to a further aspect of the invention there is provided a method of identifying a neural cell extracellular milieu extract that is effective at providing neural cell extracellular milieu directed differentiation comprising fractionating said milieu and then exposing a precursor cell population to an effective amount of at least one fraction of said milieu for an effective amount of time under conditions that support differentiation and examining said population to determine whether and/or to what degree neural cell extracellular milieu directed differentiation has taken place.

This method may be performed *in vitro* or *in vivo*.

A further aspect of the invention comprises an extract identified by the afore method.

In either of the above preferred methods for identifying an extract of the invention fractionation may be undertaken having regard to size, thus differing molecular weight components of serum may be separated and tested as per the above methods. Identification may also be pursued by ionic separation or indeed by any method known to those skilled in the art, as described in, but not limited to, the following references:

1. Hider, BC and Barlow D, Polypeptide and Protein Drugs - Production, Characterization and Formulation, (1991) Ellis Horwood, New York.

2. Hook, JB & Poste, G, Protein Design and the Development of New Therapeutics and Vaccines, (1990) Plenum Press, New York.

3. Smith, CG, The Process of New Drug Discovery and Development, (1992) CRC Press.

4. Tyle, P and Ram BP, Targeted Therapeutic Systems, (1990) Marcell Dekker, New York.

5. Palfreyman, MG, McCann, PP, Lovenberg, W, Temple, JG and Sjoerdsma, A, Enzymes as Targets for Drug Design, (1989) Academic Press, San Diego.

6. Matoren, GM, The Clinical Research Process in the Pharmaceutical Industry, (1984) Marcell Dekker, New York.

7. Smythies, JR and Bradley, RJ, Receptors in Pharmacology, (1978) Marcel Dekker, New York.

8. Smith, R B, The Development of a Medicine, (1985), Stockton Press.

Clearly, once agents have been identified that provide for either of the above types of directed differentiation it will be possible to identify, using the above techniques, further agents that block this effect and so identify antagonists to agents that are effective at providing directed differentiation.

Reference herein to precursor cells includes reference to cells which have not yet developed to their mature phenotype and ideally includes reference to cells which are in a very early stage of development and thus ideally have not begun the differentiation process and so are characterised by a lack of or shortage of tissue specific markers.

Reference herein to conditions that support differentiation will of course vary having regard to the nature of the cell population under consideration but in any event these conditions will be known by those skilled in the art. Thus, for example, for human bone precursor cells conditions which support differentiation would include exposure to dexamethasone/$(OH)_2$Vitamin D_3.

Reference herein to differentiation includes transdifferentiation where a mature cell type de-differentiates to what is considered a precursor cell type before going through a further process of differentiation to give a fully mature cell type. Alternatively transdifferentiation may occur without the said de-differentiation step.

According to a yet further aspect of the invention there is provided a method for diagnosing the existence of, susceptibility to, or predisposition towards serum directed differentiation of a precursor cell population so as to provide a serum directed phenotype comprising obtaining a test sample of blood and particularly serum from an individual to be tested and exposing precursor cells to said test sample under conditions that support differentiation and then observing the nature of the differentiated phenotype of said precursor cells.

According to a yet further aspect of the invention there is provided a method for diagnosing the existence of, susceptibility to, or predisposition towards serum directed differentiation of a precursor cell population so as to provide a serum directed phenotype comprising obtaining a test sample of precursor cells from an individual to be tested and exposing said precursor cells to blood and particularly serum, or an extract thereof, under conditions that support differentiation and then observing the nature of the differentiated phenotype of said precursor cells.

According to a yet further aspect of the invention there is provided a method for diagnosing the existence of, susceptibility to, or predisposition towards neural cell extracellular milieu directed differentiation of a precursor cell population so as to provide a neural cell extracellular milieu directed phenotype comprising obtaining a test sample of neural cell extracellular milieu from an individual to be tested and exposing precursor cells to said test sample under conditions that support differentiation and then observing the nature of the differentiated phenotype of said precursor cells.

According to a yet further aspect of the invention there is provided a method for diagnosing the existence of, susceptibility to, or predisposition towards neural cell extracellular milieu directed differentiation of a precursor cell population so as to provide a neural cell extracellular milieu directed phenotype comprising obtaining a test sample of precursor cells from an individual to be tested and exposing said precursor cells to nerve cell extracellular milieu, or an extract thereof, under conditions that support differentiation and then observing the nature of the differentiated phenotype of said precursor cells.

In the preferred methods of diagnosis where said serum or said milieu is said test sample said precursor cells comprise a human cell line such as a bone marrow stromal cell line or a human neural cell line, ideally, as herein described. Thus, the above process of observation involves assaying in order to determine the number of bone producing cells or neuronal cells or glial cells.

We also describe herein how, somewhat remarkably, adipo/osteoprogenitor precursor cells express what, up to now, was thought to be a marker for the

mature adipocyte phenotype, ie a receptor known as peroxisome profilerator-activated receptor γ (PPARγ). Given this startling observation and also the fact that ligands for this receptor induce differentiation of adipo/ osteoprogenitor precursor cells to an adipocyte phenotype we consider that this receptor and its associated ligands, have a role to play in regulating and/or controlling differentiation of at least these precursor cells. Thus reference herein to serum and/or neural cell extracellular milieu, or an extract thereof, includes reference to ligands that bind this receptor; and reference herein to agents that block serum and/or neural cell extracellular milieu directed differentiation includes reference to antagonists for these ligands and/or agents which affect the functioning of said receptor.

An embodiment of the invention will now be described by way of example only with reference to the accompanying figures wherein:

Figure 1

Fluorescence immunolocalisation of a temperature sensitive form of the simian virus-40 derived large tumour antigen expressed in immortalised human marrow stromal cell clone 7.

Figure 2

Cell growth curves for three different cell densities of immortalised human marrow stromal cell clone 7 cells over a period of 4 days. Cells were plated at densities of 2500 (open squares), 5000 (open diamonds) and 10000 per cm^2 (open circles) and incubated at the permissive temperature (33° C). Error bars provide the standard error of the mean of 4 samples for each point.

Figure 3a

Cellular alkaline phosphatase activity in immortalised human marrow stromal cell clone 7 cells in response to 7 days dexamethasone (10^{-7}M) treatment. Error bars relate to the standard error of the mean of 6 samples per point. Statistical analysis was performed by student's t-test.

Figure 3b

Osteocalcin protein expression in immortalised human marrow stromal cell clone 7 over a 4 day period. Cells were treated with a range of concentrations of 1,25 $(OH)_2D_3$ in the presence of vitamin K for 4 days. The media was then removed and osteocalcin measured by radio immunoassay. Osteocalcin levels were normalised to nanogrammes of osteocalcin per 10000 cells. Results are expressed as the mean +/- standard error of the mean of 4 samples per point. Student's t-test was used for statistical analysis.

Figure 4

Figure 4 demonstrates the effects of two agonists on cAMP levels in immortalised human marrow stromal cell clone 7. Cells were pre-treated with 1mM IBMX for 5 minutes, and then with agonist for 20 minutes. cAMP levels were quantified by ELISA, and the amounts of cAMP for each treatment compared to the control (IBMX only). Results are expressed as the mean +/- standard error of the mean of 4 samples. The statistical analysis was performed by student's t-test.

Figure 5

This figure demonstrates the effects of dexamethasone (10-7M) on the mineralisation of immortalised human marrow stromal cell clone 7. Cells were incubated for 28 days in medium containing 10% foetal bovine serum along with β-glycerophosphate at 39°C, the oncogene's non-permissive

temperature. Cells treated with dexamethasone were shown to mineralise by the von-Kossa staining procedure. Light microscopic analysis shows some nodule formation in the cultures but also evidence of mineralisation elsewhere. The mineral deposits are clearly localised to the extracellular matrix.

Figure 6

The effects of normal rabbit serum on immortalised human marrow stromal cell clone 7. Cells were incubated with either medium containing foetal bovine serum or medium containing foetal bovine serum and normal rabbit serum. In the absence of normal rabbit serum no lipid can be detected. However incubation with rabbit serum induces a dramatic change in lipid content as is demonstrated by oil-red-O staining.

Figures 7a and b

The effects of normal rabbit serum (NRS) on immortalised human marrow stromal cell differentiation. Figure 7a shows that Type IαI collagen expression (lane 2) is lost (lane 3) after one weeks treatment with NRS (123 bp ladder marker, lane 1). Conversely 7 days NRS treatment (Figure 7b) induces the expression of an early marker of adipogenesis, lipoprotein lipase (lane 2), which is not present (lane 3) in the absence of NRS (123 bp ladder marker, lane 1).

Figure 8

Demonstrates the effects of various sera type on adipogenesis of clone 7 immortalised human bone marrow stromal cells.

Figure 9

Demonstrates the effects of pre and post-menopausal serum on adipogenesis of clone 7 immortalised human bone marrow stromal cells.

Figure 10

Demonstrates the effects of 15-deoxy PGJ_2 and retinoic acid on LOP-7 cell differentiation.

The following materials and methods were used in relation to the invention described in this application.

FACTORS AFFECTING CHOICE OF DIFFERENTIATION PATHWAY IN HUMAN BONE LINES

Materials

Costar tissue culture plastics were used for cell culture (High Wycombe, UK). Minimum essential medium - alpha (α-MEM), Dulbecco's modified eagles medium (DMEM), HAM'S F12, foetal bovine serum (FCS), glutamine, penicillin/streptomycin, G418 (geneticin), Trizol reagent and *Taq* DNA polymerase were purchased from Life Technologies (Paisley, UK). Ascorbic acid, vitamin K, PTH fragment 1-34, PGE_2, forskolin, dexamethasone, polybrene, alkaline phosphatase, histochemical detection kit, para-nitrophenyl phosphate, para-nitrophenyl standard, oil-red o, trypan blue, dimethyl sulfoxide (DMSO), 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), percoll and anti-mouse IgC FITC conjugated antibody were purchased from Sigma Chemical Co. (Dorset, UK). Immu-mount was purchased from Life Sciences International (UK) Ltd. (Basingstoke, UK). The SV40 T Antigen-specific monoclonal antibody was purchased from

Cambridge Bioscience (Cambridge, UK) and the human osteocalcin RIA kit was purchased from Nichols Institute (Ca., USA). The cAMP assay kit was purchased from Amersham (Buckinghamshire, UK) and moloney leukaemia virus reverse transcriptase (M-MLV RT) and dNTPs were purchased from Promega (Southampton, UK). PCR primers for interleukins 1α, 1β, 3, 4, 6, and 8, GM-CSF and TNFα were generous gifts from Dr. W. Wishart (Sandoz Pharma, Basle, Switzerland) and collagen type 1 PCR primers were generous gifts from Dr. K. A. Hart (Zeneca, Macclesfield, UK). Primers to lipoprotein lipase were generated from published data (Gotoda et al, 1989). Amphotropically packaged retroviral vector comprising the SV40 large T antigen mutant (tsA58) and a neomycin resistance gene were a generous gift of Professor P. H. Gallimore (Institute of Cancer Studies, Birmingham, UK).

Isolation and transduction of human bone marrow stromal cells

Fresh human rib was obtained from a 68 year old female patient undergoing thoracic surgery, with no prior history of bone disease. The rib was immediately washed twice with sterile phosphate buffered saline (PBS) and all adherent tissue scraped off. The rib was then split into two halves exposing the marrow cavity, and the trabecular bone minced. Bone marrow cells were removed from the minced trabecular bone by flushing with α-MEM using a 10ml syringe fitted with a 19-gauge needle. After several flushes with media the resulting cell suspension was transferred to a universal container and allowed to stand undisturbed for 10 minutes, after which time fat deposits that had floated to the top were scraped away. The marrow derived cells were transferred to a centrifuge tube and spun at 100xg for 5 minutes to pellet the cells. The media and fat deposits were again removed and the cell pellet resuspended in 5ml of fresh media. The resuspended cells

were loaded onto a 70% percoll gradient which was spun at 460g for 15 minutes. Following centrifugation the top 25% of the gradient volume, containing the low density osteoblast precursor cells, was removed (to be finished: Haynesworth et al 1992). To this suspension an equal volume of fresh media was added and the suspension centrifuged at 100xg for 10 minutes. The resulting cell pellet was resuspended in fresh media and a single cell solution obtained by passing the cell suspension through a 19-gauge needle several times. The number of viable cells were counted using a 1% (*w/v*) trypan blue stain.

The bone marrow stromal cells were then centrifuged at 100xg for 5 minutes and the cell pellet resuspended in an appropriate volume of medium containing an amphotropically packaged retroviral shuttle vector comprising ts-SV40-T plus selection marker, and polybrene to a final concentration of 8μg/ml. The cells and media were incubated for 2 hours after which the cell suspension was again centrifuged and the cell pellet resuspended in an appropriate volume of normal medium. Cells were seeded into T75 tissue culture flasks at a final density of $4x10^7$ cells per flask. The flasks were incubated at 33°C and the cells given 3 days to adhere to the tissue culture treated plastic. Once the majority of cells had adhered to the flask, non-adherent cells were washed away and the media replaced with normal media containing G418 at a final concentration of 400μg/ml.

After 2 weeks of culture in G418 only geneticin resistant cells survived, each giving rise to a discrete colony of homogeneous SV40-T immortalised cells. The resistant colonies were recovered individually by ring cloning and cell populations expanded in tissue culture flasks. Additionally, homogeneous cell populations were derived by seeding single cells derived from individual

colonies in 96 well plates. Colony conditioned medium was required for support of the single cells during the early stages of cell number expansion.

Cell culture

A G418 resistant replicating cell clone was selected and expanded at the permissive temperature (33°C) in α-MEM culture medium containing: 1X glutamine, 1X non-essential amino acids, pen/strep, 100μg/ml G418 and 8% heat inactivated FCS (basal medium). Cells were routinely passaged twice per week, ensuring that the cells were not allowed to reach confluence. All experiments were carried out in the same basal medium unless otherwise stated.

Generation of growth curves

Cells were seeded in 24-well tissue culture plates at 2500, 5000 or 10000 cells/cm^2 and incubated at the oncogene's permissive temperature (33°C). The number of cells per cm^2 was determined at regular intervals, by using a haemocytometer to count viable cells as shown by toluidine blue exclusion, until confluence was reached.

Immunocytochemistry

Immunostaining for large-T antigen was performed after fixing subconfluent cells with methanol and acetone (1:1 ratio) at -20°C for 20 minutes. The primary IgC mouse monoclonal antibody against SV40-T was added at a 1:20 dilution in PBS and incubated at 37°C for 1 hour. Control cells were incubated in PBS alone. The cells were rinsed twice with PBS and a

secondary antibody (goat anti-mouse IgC FITC conjugated) was added to the cells at a 1:20 dilution in PBS and incubated at 37°C for a further 60 minutes. Excess antibody was then washed away with several washes with PBS and the cells overlayed with immu-mount prior to fluorescence microscopy.

Staining of mineralised matrix

The medium of postconfluent cells was supplemented with 50 μg/ml ascorbic acid and 10^{-5}M β-glycerophosphate, in the presence or absence of 10^{-7}M dexamethasone, at the non-permissive temperature (39°C) for 28 days. Cells were washed in PBS and fixed in 10% buffered formol saline for 10 minutes at room temperature. Cells were then stained by a modification of the von Kossa method (Cook 1974). Briefly cells were treated with silver nitrate and exposed to ultraviolet light for 1 hour. Excess silver nitrate was washed away with distilled water and the cells treated with 5% sodium thiosulfate for 5 minutes at room temperature. The cells were subsequently washed twice with distilled water and allowed to air dry.

Bone cell protein assays

Cells were seeded at a density of 10,000 cells per cm^2 and allowed to reach confluence, at the permissive temperature (33°C) before treatment with either dexamethasone, 1,25-$(OH)_2D_3$ or vehicle. Cells were incubated at the non-permissive temperature (39°C) and culture media, along with the appropriate agent, replenished every three days. The media was removed and stored at -80°C prior to being used for osteocalcin measurement.

Osteocalcin was measured using an immunoradiometric assay kit (Nicholl's Institute), which detects both intact osteocalcin and its large N-terminal mid-fragment. Briefly, 20μl of conditioned media or media plus known osteocalcin standards was mixed with a ^{125}I-labelled antibody to human osteocalcin and a plastic bead coated with an unlabelled osteocalcin antibody raised against an alternative site on the osteocalcin molecule. The mixtures were incubated for 3 hours at room temperature after which time the beads were washed several times with wash buffer (provided in kit). The washed beads were then placed in a gamma counter for quantification of bound ^{125}I. The control and treated samples were compared with the standard curve for quantification of osteocalcin.

Alkaline phosphatase was measured in the cell layer by a modification of the method initially described by Bessey et al (1946). Briefly, cell lysates were prepared by washing the cells several times in PBS, scraping the cell layer into 200μl of ice-cold 0.1% Triton-X 100 and subjecting the samples to mild sonication. Aliquots of the samples were then incubated with pre-warmed substrate solution (0.1M Diethanolamine, 1mM $MgCl_2$ and 2mM p-NPP pH 10.5) for 1 hour and the reaction stopped by the addition of 0.1M NaOH. Samples were quantified spectrophotometrically at 410nM and the final p-NP concentration estimated using a standard curve of known p-NP standards.

Both osteocalcin and alkaline phosphatase measurements were standardised per unit protein by determining protein content of the cell layer obtained with the Bio-Rad protein assay, based on the Bradford dye-binding procedure (Bradford 1976).

Stimulation of cAMP synthesis by parathyroid horm ne

Cells were removed from tissue culture flasks by trypsinisation and resuspended in serum free medium, containing IBMX at a concentration of 10^{-4}M, and at a cell density of 2.5 x 10^6 cells per ml. After 2 hours, 200μl of this suspension was then aliquoted into Eppendorf tubes and treated for 20 minutes with human PTH (1-34) at doses of 10^{-7} and 10^{-8}M, or PGE_2 at doses of 10^{-7} or 10^{-8}M, or Forskolin at a dose of 10^{-5}M. Following incubation the cells were boiled for 5 minutes, centrifuged and resuspended in 200μl of 0.1% Triton X-100. Samples were then freeze/thawed twice and sonicated. 20μl samples were measured for cAMP using an immunoassay kit obtained from Amersham.

Stimulation of differentiation into an adipocyte-like phenotype

Cells were seeded and cultured in media, containing 10% FCS, at the permissive temperature until approximately 60% confluent. The media was then replaced with media containing 10% rabbit serum, and the cells cultured at the non-permissive temperature (39°C) for 3 days. Following culture the cells were washed twice with PBS and then fixed with 10% buffered formol saline. Cells were stained for lipid content using the oil-red-O method as described by Cook (1974).

RT-PCR

Cells were seeded into flasks at 10,000 cells per cm^2 and cultured at the permissive temperature until confluent. Media was removed and the cells washed twice with PBS, to which Trizol reagent was added (1ml per $10cm^2$) and the RNA extracted as recommended by the manufacturers. First strand cDNA was synthesised from 5μg of RNA with 0.2μg of oligo(dT) primer in

the presence of dNTP mix (0,5mM of each) by MMLV-RT (100 units) in a final volume of 20μl. RNA and primer were incubated together at 70°C for 10 minutes and then flashed cooled on ice before adding the rest of the components, and the reaction incubated at 37°C for 1 hour. Samples were then heat treated at 95°C for 5 minutes to inactivate the enzyme. cDNA was amplified in a 100μl reaction, using the "hot-start" method described by D'Aquilla et al (1991), containing dNTP mix (0,2mM of each), specific primers (0.5.mM of each), *Taq* DNA polymerase (2.5 units), 20mM Tris-HCI (pH 8.4), 50mM KCI and 1.5mM $MgCl_2$. Primers and amplification (30 cycles) conditions used for the PCR reactions are listed in table 1.

Elucidation of adipo/osteoprogenitor precursor differentiation mechanism

To elucidate one possible differentiation mechanism for the identified human adipo/osteoprogenitor precursors, we considered factors known to regulate adipocyte expression. In particular, we studied the expression of the adipocyte marker-peroxisome proliferator-activated receptor gamma, PPARγ.

A reverse transcriptase polymerase chain reaction (rt-PCR) was performed on RNA from our human adipo/osteoprogenitor cells, using oligonucleotide primers specified for PPARγ. Remarkably, this experiment showed expression of PPARγ by the precursor cells. It has been thought until now that PPARγ, as with the other adipocyte specific nuclear receptors (the C/EBPs such as CEBPα), was only expressed in differentiated adipocytes.

Also, remarkably incubation of the cells with a known ligand for PPARγ, 15-deoxy-delta 12,14-prostaglandin J_2, induced the differentiation of the adipo/osteoprogenitor cells to the adipocyte phenotype (see Figure 10). This

indicated that 15-deoxy-delta 12,14-prostaglandin J_2, which resides within blood, is a specific factor that can modulate the differentiation of adipo/osteoprogenitor precursors.

This was of considerable interest since no-one had previously shown that PPARγ and ligands for it, may be involved in, or may be a way of, controlling osteogenic cell formation. Thus agents developed to antagonise the adipogenic effect of deoxy-delta 12,14-prostaglandin J_2, could provide for a safeguard against eg osteogporosis. Interestingly retinoic acid, which also resides in blood, has some adiogenic ability (see Figure 10) and seems to act in synergy with the PGJ_2 metabolite. It would seem then, that blocking the activity of retinoic acid should enhance bone precursor cell differentiation to the osteoblast phenotype.

The above methods used to elucidate the adipo/osteoprogenitor precursor differentiation mechanism are described either directly or indirectly in the following publications.

1) Hu, E et al, Transdifferentiation of myoblasts by the adipogenic transcription factors PPARγ and C/EBPα, Proc. Natl. Acad. Sci., USA, Vol. 92, pp 9856-9860, October 1995.

2) Kliewer, S A et al, A Prostaglandin J_2 Metabolite Binds Peroxisome Proliferator-Activated Receptor γ and Promotes Adipocyte Differentiation, Cell, Vol. 83, 813-819, 1 December 1995.

3) Forman, B M et al, 15-Deoxy-$\Delta^{12,14}$-Prostaglandin J_2 Is a Ligand for the Adipocyte Determination Factor PPARγ, Cell, Vol. 83, 803-812, 1

December 1995.

4) Greene et al, Isolation of the Human PPARγ cDNA: Expression in Haematopoetic Cells and Chromosomal Mapping, Gene Expression, Vol 4, 281-299, 1995.

FACTORS AFFECTING CHOICE OF DIFFERENTIATION PATHWAY IN RAT NEURAL LINES

Methods and Results

Neural cells from the 12 - 13 day embryonic rat raphé nuclei were immortalized by transduction with a retrovirally-packaged, temperature-sensitive oncogene (ts)SV40T (Stringer et al., 1994). After isolation and expansion of clones derived from single precursor cells at the permissive temperature of the oncogene, they were allowed to differentiate by raising the temperature to its non-permissive value. Under such basal conditions, cells from several clones exhibited similar neuronal characteristics, including neurofilament and neurone-specific enolase (NSE) positivity. In addition, markers of a serotoninergic phenotype were manifest. Inclusion of various sera (human, rabbit, foetal calf, new born calf, foetal horse, adult horse, goat and donkey) prevented the expression of this phenotype. Instead, the cells developed an astrocyte-like morphology and glial fibrillary acid protein (GFAP) expression. Delayed application of serum after the temperature shift resulted in a progressively reduced capacity of the cells to develop a full astrocytic phenotype. Similarly, withdrawal of serum after prolonging its application for more than 2 days failed to permit or provoke a return to the serotoninergic neuronal phenotype.

Foetal calf serum was fractionated using Amicon ultrafiltration tubes. Only fractions containing factors of MW > 30K were effective in eliciting the change to a glial phenotype. Micromolar concentrations of retinoic acid (some thousand times higher than the level found in 100% serum) also caused the expression of GFAP. Moreover addition of hydrocortisone at the time of the temperature shift affected the phenotype of the developing raphe cells, driving it down a glial route.

Co-culture at the non-permissive temperature of rat spinal cord background cells (ie neural cells devoid of neuronal cells) with the rate immortalized raphe precursor cell clones also led to the immortalized cells switching from the neuronal phenotype adapted under basal conditions to a GFAP-positive, flattened morphology typical of astrocytes; basal medium was used, with no added serum. GDNF (5ng/ml) did not mimic this effect. The choice of which pathway to follow is therefore governed, at least in part, by the presence of factors derived from the extracellular milieu in which neural-cells have been growing, as well as by factors present in serum. These factors may include retinoic acid and high molecular weight substance(s) found in serum.

It is concluded that individual cells in the embryonic rat raphé are capable of differentiating down at least two different routes *in vitro*. The choice of which pathway to follow is governed at least partly by factors present in serum, one of which might be retinoic acid. Differentiation along either pathway becomes increasingly difficult to reverse, until there is an apparently complete loss of the cells' ability to adopt the alternative serotoninergic neurone- or astrocyte-like phenotype.

FACTORS AFFECTING CHOICE OF DIFFERENTIATION PATHWAY IN HUMAN NEURAL LINES

Methods and Results

Neural precursor cells from the 8-week embryonic human cortex were immortalized as described above, and expanded from single cells to flask level. Cells were plated onto fibronectin-coated 24-well plates, held at 33°C for 3 - 4 days until they were about 70% confluent. Drugs were added either prior to raising the temperature to 39°C, or at the time of the temperature shift, and after a further 7 - 10 days the cultures were then fixed and immunostained. Specific factors and drugs exerted a marked effect on the resulting phenotype of the cortical cell lines. In one, for example, (HCort5b expansion) glial cell line-derived neurotrophic factor (GDNF) caused some cells to exhibit neuronal characteristics such as neurone-specific enolase immunoreactivity and a phase-bright morphology. Under identical culture conditions but in the presence of ciliary neurotrophic factor (CNTF) instead, GFAP staining became apparent in some flat, phase-dark astrocyte-like cells. In the absence of either of these two factors, no hallmarks of either glial of neuronal phenotypes were demonstrable. In another cortical cell line (HCort3b expansion), weak GFAP immunostaining was present in some cells under basal incubation conditions, but upon the removal of fibroblast growth factor (FGF) from the stock medium and the addition of dexamethasone, a large proportion of the cells developed a neurone-like morphology, and intense NSE-like immunoreactivity. FGF prevented this dexamethasone effect.

In summary, we have identified that unknown factors in some sera, known

trophic factors, and some small molecules are capable of directing the gross neuronal vs. astrocytic phenotype of human and rat neural precursors.

Results

Our experiments show retroviral transduction of primary human bone marrow stromal cells with a vector comprising the (ts)-SV40-T(A58) oncogene under the control of the MLV derived LTR promoter and neo^r conferred geneticin resistance to a series of bone cell populations derived from different sources.

Immunocytochemistry demonstrated the expression of SV40 large T-antigen in all cells within the immortalised clone (**Fig. 1**), the protein being localised to the cell nucleus, as expected. No staining was seen with the control cells which were not treated with the primary antibody. Furthermore, polyacrylamide gel electrophoresis and western blotting identified the T antigen in these cells, without the need for antigen precipitation and selection prior to blotting, giving an expected Mr for full length T-antigen of around 96Kd (data not shown).

All three of the various cell densities showed a steady increase in cell number at the permissive temperature (33° C), with the greatest growth rate seen with cells plated at a density of 10,000 cells per cm^2. From the graph (**Fig. 2**) it is evident that confluence is reached at just over 30,000 cells per cm^2, after which the cell growth rate decreased although a possible increase in the rate of cell death cannot be excluded. Approximate doubling times decreased with increasing cell density, from approximately 36 hours at the high density to 45 hours at the lowest cell density. At the non-permissive temperature (39° C) the cells did not appear to proliferate in the presence of normal

culture medium (data not shown).

Cell samples from the original clone have undergone more than 21 passages (approximately 84 population doublings). In a sample from clone 7, cells proliferated for 21 passages (approximately 84 population doublings which approximates to 10^{24} potential cells in total).

The immortalised clone was examined for both alkaline phosphatase activity (**Fig. 3a**) and osteocalcin protein expression (**Fig. 3b**), in order to determine whether the cells constitutively or inducibly express these two proteins, which are characteristic of the osteoblast phenotype.

At the permissive temperature alkaline phosphatase expression is seen, and its activity can be increased by treatment with dexamethasone at a dose of 10^{-7}M. The increase in activity due to dexamethasone is significantly retarded by the presence of active oncogene product, with activities at the permissive temperature of 0.848+/-0.21 μMoles p-NP/mg protein/hour and at the non-permissive temperature of 7.441+/-1.97 μMoles p-NP/mg protein/hour. When confluent cells were switched to the non-permissive temperature and cultured in normal medium for 7 days an increase in alkaline phosphatase activity was observed, activity at the permissive temperature being measured at 0.236+/-0.09 μMoles p-NP/mg protein/hour and at the non-permissive temperature 0.985+/-0.18 μMoles p-NP/mg protein/hour. At both the permissive and non-permissive temperatures dexamethasone treatment was associated with a significant increase in alkaline phosphatase activity ($p<0.05$ at 33°C and $p<0.01$ at 39°C). In some of our other bone marrow stroma derived immortalised clones, however, no detectable levels of alkaline phosphatase activity were seen prior to stimulation with dexamethasone or $1,25(OH)_2$

vitamin D_3.

Osteocalcin synthesis was not detected in cells cultured with basal medium for up to 3 weeks, but when 1,25 $(OH)_2$ vitamin D_3 was added to the medium along with vitamin k, osteocalcin was detected by the second week of culture. The osteocalcin response to 1,254 $(OH)_2$ vitamin D_3 was dose dependent with the lowest dose of 10^{-11}M stimulating the production of 0.41+/-0.17 ng of osteocalcin per 10^4 cells, and the top dose of 10^{-7} resulting in 6.37+/-0.55 ng of osteocalcin per 10^4 cells. As expected, exposure to dexamethasone did not lease to the synthesis of measurable quantities of osteocalcin.

cAMP responses to forskolin, PGE_2 and PTH (1-34) were studied to investigate whether these known bone agonists would induce an elevation in cAMP levels. It is evident that all three agonists have a stimulatory effect on cAMP levels (**Fig. 4**). The best response, as expected, was seen with forskolin at a dose of 10^{-5}M, which increased levels over 2-fold. Both PTH and PGE_2 gave dose dependent increases in cAMP, with PGE_2 at 10^{-9}M giving a 38.7+/-8.4% increase in cAMP compared to a 79+/-4.2% increase at 10^{-8}M, and 1-34 PTH at 10^{-9}M gave a 50+/-11.6% increase in cAMP rising to 58+/-11.02% at 10^{-8}M.

After 28 days of culture in the presence of β-glycerophosphate and with or without 10^{-7}M dexamethasone, clone 7 cells were stained using the von Kossa method to demonstrate mineralisation of the extracellular matrix. Both the treated and untreated cells mineralised their extracellular matrix, with a greater amount of mineralisation seen in the dexamethasone treated cells (**Fig 5.a**). Also, some nodule formation was seen in these cultures, but it was evident that mineralisation also occurred in non-nodule regions. Light

microscopic analysis clearly showed the presence of mineral deposits in the extracellular matrix (**Fig. 5b**).

Clone 7 cells, cultured in the presence of 10% normal rabbit serum for 3 days, showed a dramatic change in cell lipid content, as demonstrated by oil-red O staining. Untreated cells showed no detectable lipid content. However, when treated with rabbit serum, the proportion of cells showing positive staining increased to 100% (**Fig. 6a, b**). In contrast ts-SV40-T immortalised osteoblast-like cells derived from adult human trabecular bone chips could not be induced to differentiate down an adipocytic pathway (data not shown).

Treatment of the immortalised stromal cells with normal rabbit serum for one week at 39°C also induced the expression of lipoprotein lipase, an early marker of the adipocytic lineage (**Fig. 7a**). Furthermore, type I collagen expression, a marker of the osteoblastic cell lineage, was lost after incubation with 10% normal rabbit serum for 7 days (**Fig. 7b**).

Given that osteoporosis, which is characterised by an increase in marrow adipose tissue and a reduction in bone volume, tends to occur in post-menopausal females and that our results suggest that serum, or an extract thereof, provides for adipogenic switching we exposed our clone 7 immortalised human bone marrow stromal cells to rabbit sera, which we know contains an agent responsible for directing differentiations so as to provide adipocytes, pre-menopausal human female serum, which we speculate contains relatively little or no such agent, and post-menopausal human female serum which we speculate may contain such an agent. The results are shown in figures 8 and 9.

In figure 8 it can be seen that, as predicted, exposure of our human cell lines to rabbit serum resulted in, approximately, a 90% production of adipocytes. In contrast, exposure of the same cell line to pre-menopausal human female serum resulted in the production of only, approximately, 10% adipocytes, suggesting that the agent responsible for adipocyte switching was either less active, present in a lower amount, or absent. Exposing the same cell line to post-menopausal human female sera resulted in the production of approximately 40% adipocytes, suggesting that serum directed differentiation to the adipocyte phenotype had taken place and thus the agent responsible for adipocyte switching was either more active in this serum or present in a greater amount.

Referring to figure 9 it can be seen that when essentially the same experiment was repeated using sera from a group of pre-menopausal females and a group of post-menopausal females the same results were obtained.

With reference to Figure 10, it can be seen that the adipo/osteoprogenitor precursors differentiate down a pathway which involves the peroxisome profilerator-activated receptor γ PPARγ. The role of this receptor in differentiation is remarkable given that, conventionally, this receptor is thought to be a marker of a differentiated and thus fully mature phenotype. Accordingly, our data suggests that agents which activate/deactivate PPARγ will have a role to play in the differentiation of these precursor cells.

This data suggests that there is in sera an agent that is responsible for directing differentiation and when human bone marrow stromal cells are exposed to said agent an adipocyte phenotype is favoured.

Our results clearly show that using human or animal cell lines that have undergone retroviral transduction with a vector expressing a modulateable form of SV40-T we are able to provide precursor cells which, at the oncogene's permissive temperature, can be replicated into a homogeneous population of precursor cells. These cells can then be used to study differentiation. Using these cells we have been able to show that precursor cells may be multipotential and thus depending upon the nature of the differentiation process they undertake can differentiate into more than one phenotype. Thus, for example, we have shown that bone precursor cells can be made to differentiate to produce either bone cells or adipocytes, nerve precursor cells can be made to differentiate to produce either neuronal cells or astrocytes. Our results indicate that the choice of pathway to follow is governed at least by soluble factors present in serum and/or nerve cell extracellular milieu. We consider that low molecular weight soluble factors are involved and that furthermore these molecular weight soluble factors are likely to be carried by large molecular weight carrier proteins. We also have shown that differentiation along alternative pathways becomes increasingly difficult to reverse as the differentiation process progresses until there is an apparent complete loss of ability to adopt to anything other than the committed pathway.

Our findings and thus the invention described in this application has enormous technical significance in that we have established: that precursor cells can develop along more than one pathway; how this development is directed; and how this development may be regulated.

CLAIMS

1. A method of regulating or controlling the differentiation of precursor cells to at least one specific phenotype comprising exposing said cells to blood and particularly serum, or an extract thereof and/or neural cell extracellular milieu, or an extract thereof, under conditions that support differentiation so as to selectively drive differentiation with a view to providing a pre-selected mature phenotype.

2. A method according to Claim 1 wherein said precursor cells are exposed, alternatively, or in addition, to an agent that blocks the activity of said serum or said milieu.

3. A pharmaceutical composition comprising an effective amount of blood and particularly serum, or an extract thereof, and/or neural cell extracellular milieu, or an extract thereof, for directing differentiating of selected precursor cells to at least one specific phenotype.

4. A pharmaceutical composition comprising an effective amount of an agent that blocks the activity of blood and particularly serum, or an extract thereof, and/or neural cell extracellular milieu, or an extract thereof, so as to provide for serum or neural cell extracellular milieu independent differentiation of the selected precursor cells to at least one serum or neural cell extracellular milieu independent phenotype.

5. A method of identifying agents that are effective at preventing or interfering with serum directed differentiation and/or neural cell extracellular milieu directed differentiation comprising: exposing a precursor cell

population to an effective amount of

a) blood and particularly serum, or an extract thereof, and/or an effective amount of neural cell extracellular milieu, or an extract thereof; and/or

b) a test agent,

for an effective amount of time under conditions that support differentiation and examining said population to determine whether and/or to what degree serum, or neural cell extracellular milieu directed differentiation has taken place.

6. A method according to Claim 5 wherein said population is a cell-line.

7. A method according to Claim 6 wherein said cell-line is of human origin.

8. A method according to Claim 7 wherein said cell-line is a human bone marrow stromal cell-line.

9. A method according to Claim 7 wherein said cell-line is a human neural cell-line.

10. A method of identifying agents that are effective at enhancing serum and/or neural cell extracellular milieu directed differentiation comprising: exposing a precursor cell population to an effective amount of

a) blood and particularly serum, or an extract thereof, and/or an effective

amount of neural cell extracellular milieu, or an extract thereof; and/or

b) a test agent,

for an effective amount of time under conditions that support differentiation and then examining said population to determine whether and/or to what degree serum or neural cell extracellular milieu directed differentiation has taken place.

11. The agents identified by the methods of Claims 5 to 10.

12. A method of identifying a blood and particularly a serum extract that is effective at providing serum directed differentiation comprising fractionating said blood and particularly serum and then exposing a precursor cell population to an effective amount of at least one fraction of said serum for an effective amount of time under conditions that support differentiation and examining said population to determine whether and/or to what degree serum directed differentiation has taken place.

13. A method of identifying a neural cell extracellular milieu extract that is effective at providing neural cell extracellular milieu directed differentiation comprising fractionating said milieu and exposing a precursor cell population to an effective amount of at least one fraction of said milieu for an effective amount of time under conditions that support differentiation and examining said population to determine whether and/or to what degree neural cell extracellular milieu directed differentiation has taken place.

14. The extract identified by the method of claims 12 or 13.

15. A method for diagnosing the existence of, susceptibility to, or a predisposition towards serum directed differentiation of a precursor cell population so as to provide a serum directed phenotype comprising obtaining a test sample of blood and particularly serum, or an extract thereof, from an individual to be tested and exposing precursor cells to said test sample under conditions that support differentiation and then observing the nature of the differentiated phenotype of said precursor cells.

16. A method for diagnosing the existence of, susceptibility to, or a predisposition towards serum directed differentiation of a precursor cell population so as to provide a serum directed phenotype comprising obtaining a test sample of precursor cells from an individual to be tested and exposing said precursor cells to blood and particularly serum, or an extract thereof, under conditions that support differentiation and then observing the nature of the differentiated phenotype of said precursor cells.

17. A method for diagnosing the existence of, susceptibility to, or a predisposition towards neural cell extracellular milieu directed differentiation of a precursor cell population so as to provide a neural cell extracellular milieu directed phenotype comprising obtaining a test sample of neural cell extracellular milieu, or an extract thereof, from an individual to be tested and exposing precursor cells to said test sample under conditions that support differentiation and then observing the nature of the differentiated phenotype of said precursor cells.

18. A method for diagnosing a method for diagnosing the existence of, susceptibility to, or predisposition towards neural cell extracellular milieu directed differentiation of a precursor cell population so as to provide a neural

cell extracellular milieu directed phenotype comprising obtaining a test sample of precursor cells from an individual to be tested and exposing said precursor cells to nerve cell extracellular milieu, or an extract thereof, under conditions that support differentiation and then observing the nature of the differentiated phenotype of said precursor cells.

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44

Application N : GB 9610809.7
Claims searched: 1-18

Examiner: Nicola Curtis
Date of search: 28 August 1996

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Search Report under Section 17

Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK Cl (Ed.O): C6F (FHC1; FX)

Int Cl (Ed.6): C12N 5/00

Other: ONLINE: WPI; BIOTECH/DIALOG

Documents considered to be relevant:

Category	Identity of document and relevant passage		Relevant to claims
E,X	WO 96/16162 A1	(National Jewish Center For Immunology and Respiratory Medicine) (See page 12, line 14 to page 13, line 28; page 17, line 25 to page 18, line 23; page 19, line 12-18; page 20, line 5-11; Examples 7 & 9)	1
E,X	WO 96/04368 A1	(Dalhouse University) (See example 3; page 13, line 26 to page 14, line 10)	1, 2, 5, 10
X	WO 94/09119 A1	(Neurospheres Ltd.) (See page 10 & 11)	1, 3
X	WO 94/04658 A1	(Schering Corp.) (See page 4, line 11 to page 5, line 4)	1, 3
X	WO 93/17696 A1	(The Regents of the University of Michigan) (See claims 1-3; paragraph bridging pages 5-6)	1
X	WO 93/17045 A1	(Alliance Pharmaceutical Corp.) (See Example 14; page 3, lines 9-19)	1
X	WO 93/05807 A1	(New England Deaconess Hospital) (See claims 1, 22; page 5, lines 23-28)	1, 3, 10, 11

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.

The Patent Office

45

Application No: GB 9610809.7 **Examiner:** Nicola Curtis
Claims searched: 1-18 **Date of search:** 28 August 1996

Category	Identity of document and relevant passage	Relevant to claims
X	WO 92/01039 A1 (Dana-Farber Cancer Institute) (See page 9, line 7 to page 10, line 10)	1, 3
X	WO 91/09936 A1 (Hana Biologics) (see page 23-24)	1
X	WO 91/07992 A1 (Monash University) (See 3, paragraph 1; paragraph bridging pages 3 & 4)	1, 3
X	MOLECULAR ENDOCRINOLOGY, Vol. 7, No. 2, 1993, Gazit et al., "Modulation of expression and cell surface binding of members of the transforming growth factor-β superfamily during retinoic acid-induced osteoblastic differentiation of multipotential mesenchymal cells", pages 189-198 (see final paragraph on page 190)	1
X	PROC.NATL.ACAD.SCI., Vol. 74, No. 3, 1977, Hoffman et al., "Endotoxin-induced serum factor controlling differentiation of bone-marrow-derived lymphocytes", pages 1200-1203 (see Discussion)	1

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
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&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.